Volume 193, number 2 FEBS 3180 December 1985

Cyanogen-induced γ-glutamyl to imidazole cross-link in carbonic anhydrase

A unique mode of inhibition

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Received 15 October 1985

The irreversible inhibition of carbonic anhydrase by cyanogen occurs by a unique mechanism. Cyanogen is an affinity label: it behaves like a carbodiimide and produces an intra-molecular cross-link without being incorporated. The nucleophile-labile cross-link is formed between a γ-COOH of a Glu and an imidazole of a His with a 1:1:1 stoichiometry with the enzyme. The deletion of ~1 Glu and ~1 His was noted by amino acid analysis of enzymatically hydrolyzed carbonic anhydrase. The modified Glu was converted to 2,4-diaminobutanoic acid and quantitated by amino acid analysis. The presence and quantity of modified His was supported through high-resolution proton NMR analysis.

Carbonic anhydrase *Enzyme inhibition* *Cross-linking* *Cyanogen*

1. INTRODUCTION

The hydratase and/or esterase activities of 3 carbonic anhydrases are irreversibly inhibited by cyanogen [1–3]. We report here that the cyanogen-induced modification [1] is active site directed [2], stoichiometric [3], involves carbodiimide-type carboxyl activation, and [4] is not accompanied by $[^{14}C]C_2N_2$ incorporation into the enzyme.

2. MATERIALS AND METHODS

2.1. *Bovine carbonic anhydrase*

Preparation was carried out by the procedures of Osborne and Tashian [4] and Bergenhen et al. [5] from bovine erythrocytes.

2.2. *Assays*

Hydratase assays were performed according to Khalifah [6] as modified [2]. Esterase activity was measured by the procedure of Pocker and Stone [7] as modified [2].

2.3. *Cyanogen treatment*

C_2N_2 was obtained from Matheson Scientific. Procedures were as described [2].

2.4. *[^{14}C]Cyanogen preparation*

The procedure of Kim and King [8] was adapted to a microscale [9]. $Ag^{14}CN$ (California Bionuclear) was pyrolyzed in a Vycor vessel at 400°C and the $[^{14}C]C_2N_2$ trapped in a liquid-N_2-cooled limb of the evacuated, all-glass system. The original specific activity of 30.6 Ci/mol $Ag^{14}CN$ was diluted as needed.

2.5. *Amino acid analysis*

Standard single-column analyses were performed with ninhydrin detection on a Beckman 119 analyzer. The amino acid analyses were performed by 2 methods: (i) Acid hydrolyzates: the carbonic anhydrase (CA) samples were subjected to 6 N HCl, 110°C, for 24 h or 3 N *p*-

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Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/85/$3.30

toluenesulfonic acid, 110°C, 24 h; (ii) Enzymic digests: the procedures of Chin and Wold [11] and Royer et al. [12] were used.

2.6. *γ-Glutamyl conversion to 2,4-diaminobutanoic acid*

CA (~5×10^{-5} M) was treated simultaneously with 5×10^{-3} M C_2N_2 and NH_2OH at room temperature for 2 h. The control unmodified CA at ~5×10^{-5} M was treated with only 5×10^{-3} M NH_2OH. Excess NH_2OH was removed by dialysis. After dinitrophenylation the Lossen rearrangement was carried out by the procedure of Wilcox [10].

2.7. *Fluorescence measurements*

Fluorescence spectra were taken on an Aminco Bowman spectrofluorometer after the appropriate dilution. Concentrations of both dansylamide (DNSA) and C_2N_2-treated or untreated CA were 1×10^{-6} M. Excitation wavelengths were 290 nm (Trp) and 320 nm (DNSA).

2.8. *NMR*

High-resolution ^{1}H-NMR measurements were made on a Nicolet NMC 300 MHz spectrometer. C_2N_2-treated and control CAs were dissolved in D_2O (approx. 10 mg/ml).

3. RESULTS AND DISCUSSION

Where affinity labels have been used (see [13]) the various mammalian CAs show characteristic patterns; some of these percentage inhibition values have been compared with values from various laboratories [2]. The main point is that C_2N_2 is effective at low concentrations – often lower than other inhibitors. Comparison of time-concentration data (see [2]) suggests that C_2N_2 under the conditions described acts more rapidly at lower concentrations than most of the more potent irreversible inhibitors reported to date. Several effective affinity labels prior to this work are analogs of HCO_3^- and some compete with it during their first, reversible interaction, prior to irreversible inhibition. However close an analog a given inhibitor may or may not be, there appears to be a unique pattern of altering kinetic parameters and sites of binding dependent on the CA used in each case.

The time course of the inhibition was examined. The esterase activity of the bovine CA (BCA) is 54% inhibited in 5 min in the presence of 1 mM C_2N_2. The inhibition rises to 74% in 15 min, 85% in 25 min, and to a final ~97% after >1 h.

A useful relationship between k_{obs} and $1/C$ for affinity labeling has been noted [14]. An affinity labeling probe should exhibit a binding step prior to covalent modification of the active site. This is demonstrated by a linear relationship between

Table 1

Amino acid analyses

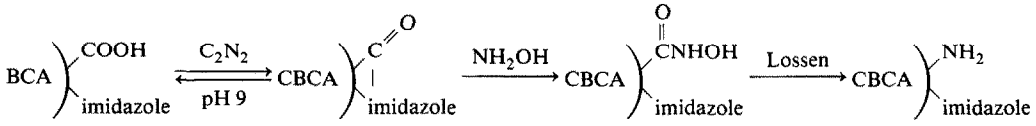
Amino acid	*p*-Toluenesulfonic acid hydrolysis[a] BCA	Reported analysis BCA	Amino acids	Enzyme digestion BCA	Enzyme digestion CBCA	Reported analysis BCA
Asx	32.6	32	Asp	18.0	17.9	18
Glx	24.6	24	Glu	12.9	11.6	11 or 12
Gly	20.5	20	Gly	19.7	19.8	20
Ala	16.7	17	Ala	17.6	17.4	17
Val	19.5	20	Val	20.8	20.8	20
Tyr	8.6	8	Tyr	8.6	8.3	8
Phe	11	11	Phe	11	11	11
His	11.2	11	His	11	9.8	11
Lys	17.9	18 or 19	Lys	19	17.7	18 or 19

[a] For the amino acids shown, essentially identical results were obtained by hydrolysis with 6 N HCl

$1/k$(obs) and $1/C(C_2N_2)$ within the concentration range that we were able to examine [9]. The linear relationship between the inverse observed rate 'constant', k, and concentration, C, is indicative of reversible binding of C_2N_2 prior to covalent modification [14].

Amino acid analysis of BCA and cyanogen-treated BCA (CBCA) acid hydrolyzates shows no difference (table 1). Amino acid analysis of total enzyme digest (as in [12]; table 1) showed a diminution by one each of Glu and His.

The lability of the modification to nucleophiles was demonstrated [1] with hydroxylamine and [2] with raised pH. The former provided further substantiation that a single Glu is modified. Treatment of the CBCa with NH_2OH to cleave the labile putative γ-COOH to imidazole link was followed by carrying through the Lossen arrangement by a modification of the procedures of Wilcox [10]. The results of 4 modifications and analyses gave 0.9, 1.4, 1.3 and 1.3/mol where the resultant average indicates 1.2 mol 2,4-diaminobutanoic acid (DAB) formed per mol CBCA. Control BCA showed no detectable DAB. On the other hand, incubation of the cyanogen-inhibited enzyme, CBCA, at pH 9 gave essentially complete recovery of enzymatic activity. The postulated reactions are:



Amino acid analysis of the enzymatic digest, but not of the acid hydrolyzate, showed a reduction of ~1 His/mol carbonic anhydrase. Further confirmation that His is involved comes from the ^{1}H-NMR analysis where the resonances assignable to a C-2 and C-4 proton of a single His imidazole are shifted. 300 MHz ^{1}H-NMR studies of both BCA and CBCA have been undertaken. The spectrum of the unmodified protein is typical except for 4 resonances found in the 12–15 ppm spectral region (fig.1a). These resonances most likely arise from the C-2 protons of the histidines bound to or near the Zn^{2+}. Modification of BCA with cyanogen apparently shifts the resonance at 12.3 ppm from this region. In addition, the peak at 8.7 ppm of the native protein tentatively assigned to the C-4 proton of a histidine is also shifted (fig.1b). These results are in agreement with the postulated mode of inhibition of cyanogen by modifying a single residue. In the upfield region (0 to −2 ppm) (fig.1c) where the ring current shifted resonances are found, one or more resonances are shifted downfield upon cyanogen treatment. This shift could either be caused by the decrease in aromaticity of an aromatic residue by cyanogen modification or an increase in the distance between the ring current shifted residue and an aromatic residue. The latter case seems unlikely because of the lack of noticeable shifts in other spectral regions which would be expected if the cyanogen treatment induced a conformational change in the entire protein.

$[^{14}C]C_2N_2$ is not incorporated at a detectable level in the maximally inhibited enzyme. However, a moderately involatile low molecular mass ^{14}C by-product, presumably $NCCONH_2$, showing the expected stoichiometry associated with a carbodiimide-type reaction was demonstrated as follows:

(1) incubate CA + $[^{14}C]C_2N_2$, 90 min, room temperature; (2) remove excess C_2N_2 in stream of N_2 passed over 400 μl reaction mixture for 10 min; (3) count ^{14}C in solution; (4) dialyze with centricon membrane filters; (5) count ^{14}C in retentate; (6) lyophilize.

At step 3 an approx. 1:1 stoichiometric ^{14}C relationship was seen. However, all of the ^{14}C disappears upon dialysis; i.e., a negligible ^{14}C remains at step 5. The CBCA in the retentate shows no regaining of activity upon simple dialysis at a neutral pH. The modified CBCA remains inactive for many days when stored near neutrality at room temperature.

The modified CA protein shows no measurable change in pI nor M_r by SDS-PAGE. Other proteins, viz. lysozyme, human serum albumin, penicillinase, hemoglobin, histones, and chro-

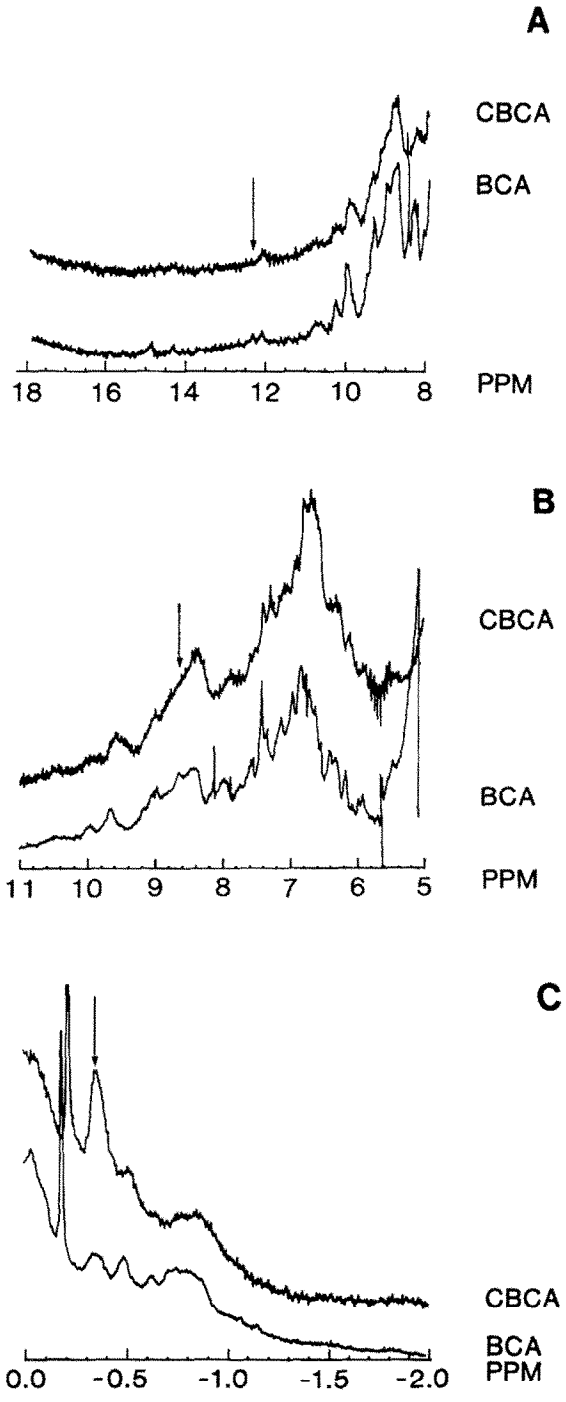


Fig.1. NMR spectra. Unmodified BCA and CBCA. The upper traces are always of CBCA. The arrows indicate resonances that are judged to be significantly different between the 2 in each case.

matin, treated under the same conditions show no loss of enzymatic activity (lysozyme, penicillinase) and little or no detectable physical changes, but at 10^2–10^3-times higher concentrations of C_2N_2 show loss of activity, large changes in p*I* and inter-subunit cross-linking where there is more than one subunit (Tharp and Day, in preparation). Treatment of CA at pH ~10 restores enzymatic activity; treatment with NH_2OH does not, but affords a substrate for the Lossen rearrangement. We have recently demonstrated that humans exposed to cyanogen have produced antibodies to human serum albumin treated with cyanogen in vitro (R. Tharp, R.A. Day and L. Bernstein, unpublished).

Fluorescence energy transfer (resonance energy transfer, Förster transfer) is dependent on the parameters R^{-6}, J (spectral overlap) and κ^2 (orientation factor). It is well documented to occur between Trps and DNSA in CA [15]. It would be expected that a change in any one of the parameters could alter the transfer and show itself as a change in the proportion of intrinsic Trp emission/DNSA emission. The important result obtained here is that the cyanogen-treated enzyme, CBCA·DNSA complex shows a ~50% reduction in the intrinsic Trp quenching when compared with the control BCA·DNSA complex while maintaining stoichiometric binding (1:1). Without DNSA, BCA and CBCA showed no significant difference between them in Trp fluorescence. Translation of these data into exact structural changes is not possible; however, it strongly implies an altered binding of the DNSA without reorientation of the Trp indole side chains. It may be orientational (κ^2), or spacing (R), or both. But clearly this interaction has been perturbed. Circular dichroic spectra [9] in the Trp region (250 to >300 nm) showed no significant change upon C_2N_2 treatment; the alteration in the resonance energy transfer efficiency of ~50% then would appear to arise from a perturbation in the DNSA binding.

In conclusion, we have provided evidence that the inhibition of CA [1–3] is an active site or substrate-binding site directed modification and is accompanied by specific changes in covalent structure, viz. γ-Glu to His imidazole linkage. The unique aspect of the modification is that C_2N_2 behaves as if it were an affinity label with its rapid irreversible inhibition at low concentrations and is not itself incorporated.

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